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(54) Title: HETEROLOGOUS EXPRESSION SYSTEM COMPRISING A B. SUBTILIS PROMOTER

(57) Abstract: The present invention relates to expression cassettes suitable for the expression of a heterologous gene. Furthermore, the invention relates to recombinant plasmids comprising such expression cassettes. Also, the invention relates to host cells comprising such cassettes or plasmids. Another embodiment relates to promoters for use in an expression cassette and in heterologous expression systems. Finally, an embodiment relates to methods for the expression of a heterologous gene.

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Novel bacterial expression system

The present invention relates to expression cassettes, to recombinant plasmids comprising such expression cassettes, to host cells comprising such cassettes or recombinant plasmids, to promoters for use in an expression cassette and use in heterologous expression systems and to methods for the expression of a heterologous gene.

Bacterial heterologous expression systems are universally used for the expression of a variety of different heterologous genes. A heterologous gene is considered to be a gene that does not originate from *Bacillus subtilis* or, if originating from *Bacillus subtilis*, is not or not solely under the control of its native promoter. It may be a gene of bacterial origin, found in either the bacterial cell that is also used as the host for the expression system (e.g. in the case that over-expression of a native gene is needed) or from another species. It may equally well be a gene of eukaryotic origin. Bacterial heterologous expression systems may have a few known disadvantages over eukaryotic heterologous expression systems, but for the expression of many heterologous genes of both prokaryotic and eukaryotic origin, they provide very versatile and inexpensive systems. With regard to the host cells of bacterial expression systems, a difference is made between the Gram-positive and Gram-negative bacteria. Gram-negative bacteria have both an inner and an outer membrane. The outer membrane sometimes interferes negatively with transportation of secreted proteins over the cell membrane. Gram-positive bacteria do suffer to a much lesser extent from this problem, because they lack the outer membrane. A few randomly chosen examples of Gram-positive bacteria suitable as host cell for heterologous expression systems are e.g. bacteria belonging to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Bacillus*, *Sarcina*, *Ruminococcus* or *Listeria*.

A problem connected with all heterologous expression systems is the fact that (over)-production of the heterologous expression product frequently interferes in a negative way with the viability or growth characteristics of the host cell in which it is expressed. Thus, when choosing a bacterial expression system, it has to be decided first, if the heterologous protein to be expressed is detrimental or even toxic for the host cell. If this is the case, the heterologous expression system would preferably have the option of switching the heterologous expression on or off. But even if the heterologous protein to be expressed is known to be only slightly or even not toxic at

all for the host cell, continuous expression of the heterologous gene after the moment that growth reaches the stationary phase would finally kill the host cell. In such a case, an expression system would be preferred that continuously synthesizes the desired heterologous protein, but only to a level that is directly linked to the growth rate of the bacterium: if the growth rate slows down or halts, the heterologous expression level should preferably slow down or halt as well.

It is an objective of the present invention to provide a highly versatile expression system that offers a solution to the problems indicated above.

Surprisingly, it was found now that two genes coding for Single-stranded DNA Binding Proteins (SSB) found in i.a. the *B. subtilis* genome have promoter sequences that do have the characteristics that make them very suitable for a heterologous expression system with one or both of the desired characteristics.

One SSB-like gene, further referred to as *ssb*, maps at 358.6° of the *B. subtilis* genome and is flanked by the *rpsF* and *rpsR* genes coding for the ribosomal proteins S6 and S18, respectively (Figure IA). A structure similar to Rho-independent transcriptional terminators is situated downstream of the *rpsR* gene. Since no terminator-like structure was found between the genes *rpsF, ssb* and *rpsR*, it is likely that these genes belong to an operon. The second *ssb*-like gene, *ywpH*, maps at 319.4 'and is flanked by another gene of unknown function (*ywpG*) and the *glcR* gene coding for a regulator involved in carbon catabolite repression (Figure 1B). Full details of the location and the sequence of the genes mentioned have been given in F. Kunst et al., Nature 390: 249-256 (1997).

Transcriptional fusions of the potential promoter-containing fragments with a promoter less lacZ gene were constructed and integrated into the *B. subtilis* chromosome at an ectopic site. An ectopic site is a site at which the gene is not found in the wild-type bacterium. Upstream regions of the *ssb* and *ywpH* genes, indicated as S 1, S2, R1 and Y1 were amplified by PCR using chromosomal DNA of *B. subtilis 168* as template. The PCR fragments were cloned into a promoter-screening vector and introduced via transformation into *B. subtilis 168,* selecting for kanamycin resistant transformants. The transformants were screened for an amylase-deficient phenotype to confirm that the construct had integrated at the selected ectopic site, i.e. the *amyE* locus. (The *amyE* locus is merely an example of a

suitable integration site, because the *amyE* gene is a non-essential gene, and can thus safely be used for the insertion of another gene.

The blue or white phenotypes of the resulting strains BIV7 (S1), 17 (S2), *8 (RI)*, and 12 (Y1) on rich (TY) and minimal medium (MM) agar plates revealed promoter activity only for the constructs in strain BIV 17, *8*, 12 and 13, but not in BIV7. In BIV12, promoter activity was detected only in MM indicating a medium-dependent expression of the gene *ywpH* (Figure 1). Whereas *ywpH* was found to be transcribed from a promoter directly upstream of the gene, no promoter activity could be detected from the S 1 fragment comprising the 295 bp immediately upstream of the *ssb* start codon. However, promoter activity was detected from the S2 fragment containing also the complete *rpsF* gene and 274 bp upstream of its start codon indicating that *ssb* is the second gene of an operon, which is constituted of the *rpsF*, *ssb*, and *rpsR* genes. Clearly promoter activity was found also from the smaller RI fragment comprising a 274 bp upstream of the *rpsF* start codon only. These results indicate that *ssb is co*transcribed and co-regulated with genes coding for ribosomal proteins, thereby coupling the regulation of protein synthesis and DNA metabolism.

In order to study the expression of *ssb* and *ywpH* in more detail, strains containing the S1-, R1- and Y1-lacZ fusions (BIV7, *8* and 12), respectively, were grown in TY and MM and ß-galactosidase activity was examined as described by Miller et al. (Miller, J. H. 1982. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Whereas no expression could be detected from the S1 fragment under the conditions employed, the *ssb* operon appeared to be strongly expressed from the *rpsF*-promoter in both rich and minimal media. The highest values (between 200 and 300 Miller units per OD) were reached during exponential growth (Figure 2A, B). When cells stopped growing the expression dropped. There is still expression in the stationary phase but on a much lower level.

In conclusion these results show directly growth-related characteristics of expression behavior of the *ssb* operon, and thus of the *rpsF*-promoter. Therefore, this promoter clearly is the promoter of choice for the expression of heterologous genes encoding non-toxic or slightly toxic proteins. The level of expression is directly linked to the growth rate of the bacterium. The protein encoded by the heterologous gene will be expressed only during logarithmic growth. If the growth rate of the bacterium slows down, e.g. as a result of decreased amounts of nutrients, the synthesis of the protein encoded by the heterologous gene also decreases, thus avoiding growth retardation

or even killing of the host cell, i.e. the bacterium, by over-production of the heterologous gene.

One of the advantages of such an expression system is, that the host cells remain viable. Further expression can very easily be obtained by just adding more medium to the cells, regardless the fact that the cells may have temporarily reached at any moment in time the stationary phase.

In contrast, no expression of the *ywpH* gene could be detected in cells of *B. subtilis* BIV12 grown in TY and only very low expression was observed in exponentially growing cells in MM. However, expression of *ywpH* increased significantly, when cells growing in MM entered the stationary phase and reached its highest level within two hours thereafter (Figure 2C).

In conclusion: the *ywpH* promoter is only expressed during the stationary phase in MM. This promoter would clearly be the promoter of choice for the production of proteins that are toxic for the host cell, because during logarithmic growth of the cells, growth is not at all hampered by expression of toxic heterologous protein. Genes encoding such proteins can safely be cloned downstream of the *ywpH* promoter since the cells can be grown in MM or rich medium to a high density without any heterologous gene expression. Only as soon as the stationary phase is reached and cells are grown in MM, the heterologous toxic protein will be synthesized. Even more efficiently, cells can be grown in rich medium to high densities, i.e. the stationary phase, followed by further culturing in MM. This will then trigger the synthesis of the toxic protein. If needed, a small sample of cells can be taken out during logarithmic growth phase and kept frozen for use in further cultures.

The basic element of a heterologous expression system is the combination of the promoter and the heterologous gene to be expressed. This combination is in principle sufficient for obtaining the heterologous expression product, i.e. the protein encoded by the heterologous gene, as expressed under the influence of the *rpsF*-promoter or the *ywpH*-promoter.

This basic element will be further referred to as an expression cassette.

It should be clear however, that usually this expression cassette will subsequently be cloned into a plasmid, and/or be inserted in the genome of the host.

The recombinant plasmid carrying the expression cassette can be used for the transformation of the host bacterium. If a high-copy plasmid is chosen, this will generally lead to an enhanced expression, due to the fact that several copies of the expression cassette will be expressed at the same time in the host. This allows for a

further fine-tuning of the amount of expression product made. Such high-copy plasmids are known in the art. Instead of maintaining the expression cassette on a plasmid in the host, the expression cassette can be integrated, in one or more copies, in the genome of the host cell, e.g. through the process of homologous recombination. A combination of the two systems is also feasible.

Thus a first embodiment of the present invention relates to an expression cassette comprising a promoter which is obtainable from the *Bacillus subtilis* genome wherein the promoter is located in the region between the stop codon of the *yyaF*-gene and the start codon of the *rpsF*-gene.

Only one promoter is found in this region, and therefore the fact that the promoter according to the invention is located in the region between the stop codon of the *yyaF*-gene and the start codon of the *rpsF*-gene is an unequivocal identification of the promoter.

As mentioned above, full details of the location and the sequence of the genes mentioned here have been given in F. Kunst et al., Nature 390: 249-256 (1997).

Genomes of microorganisms whose complete genome sequence is known were screened for SSB homologues and their gene organization using the NCBI database (http://www.ncbi.nhn.nih.gov). At this moment 69 complete sequences of bacterial genomes are available including 59 different species. Of these, 23 species show the *ssb* gene flanked by the *rpsF* and *rpsR* genes as was observed for *B. subtilis*. These species mainly belong to the taxonomic group of the Firmicutes, like *B. subtilis* itself, to the Spirochaetales, Thermotogales and Aquificales.
This search revealed that the gene organization *rpsF ssb rpsR* is commonly found in these taxonomic groups.

The sequence of this region in *Bacillus subtilis* is given in figure 3. The desired promoter region, which is described in more detail below, can now easily be found on the genome of *Bacillus subtilis*, and subsequently be synthesized and cloned with the help of standard PCR techniques, well-known in the art. Such PCR- and cloning techniques are e.g. described in standard laboratory manuals such as in Dieffenbach & Dreksler; PCR primers, a laboratory manual. ISBN 0-87969-447-5 (1995) and in Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6).

The promoter of the expression cassette according to the present invention is obtainable from *Bacillus subtilis* as mentioned above, but was shown, as also stated above, to be present in the taxonomic group of the Firmicutes, like *B. subtilis* itself, the Spirochaetales, Thermotogales and Aquificales. It can therefore easily be located and cloned from these taxons by screening the genome of any of the bacteria belonging to these taxons, using the sequence given in figure 3 as a probe. Such screening is also a standard technique, known in the art for years now, and also described in Dieffenbach & Dreksler; PCR primers, a laboratory manual. ISBN 0-87969-447-5 (1995) and in Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6).

Therefore, promoters as described in the invention obtained from any of the taxonomic groups mentioned above are considered to be within the scope of the present invention.

It is clear that any of the taxonomic groups mentioned above are also suitable for use as a host cell for expression cassettes according to the invention, because the promoter is present in, and thus recognized in these taxonomic groups.

Promoter sequences can be recognized by a few motives, such as their specific so-called –35 site, -10 site and the spacer between these sites, as well as the fact that at a certain distance from these sites of (very roughly) 50-65 nucleotides downstream of the –10 site a ribosome binding site motive is found. The distance between –10 site and RBS is however far from critical.

In a preferred form of this embodiment, the *rpsF* promoter of the heterologous expression system according to the invention has the following characteristics:

a) a nucleotide sequence TATAAT, preferably GTATAAT, TATAATA or GTATAATA, more preferably GGTATAAT, TATAATAT or GGTATAATAT at the –10 site,

b) a nucleotide sequence TTGTAA, preferably GTTGTAA, TTGTAAA or GTTGTAAA, more preferably AGTTGTAA, TTGTAAAG or AGTTGTAAAG at the–35 site,

c) a spacer of 17 +/- 2 nucleotides, preferably 17 +/- 1 nucleotides, more preferably 17 nucleotides.

A spacer is understood to be the nucleic acid sequence separating the –35 sequence TTGTAA from the –10 sequence TATAAT. This spacer varies in length between 15 and 19 nucleotides.

In a more preferred form of this embodiment, the promoter comprises a nucleotide sequence as depicted in SEQ ID NO 1.

In principle, the Ribosome Binding Site (RBS) to be used is not critical, especially because it is not an actual part of the promoter as such. Nevertheless, the RBS as found between the *rpsF*-promoter and the start codon of the *rpsF* gene would be a preferred RBS.

Therefore, in an even more preferred form, the promoter comprises a nucleotide sequence as depicted in SEQ ID NO 2. This sequence differs from the sequence of SEQ ID NO 1 in that it additionally comprises the sequence downstream of the –10 region to include the Ribosome Binding Site (RBS) preceding the *rpsF* gene.

In order to obtain the most native form of the *rpsF* promoter, it is preferred to also use the region between the RBS and the start-codon (preferably including the start codon) of the *rpsF* gene.

Thus, in a most preferred form, the promoter according to the invention comprises a nucleotide sequence as depicted in SEQ ID NO 3. This sequence differs from the sequence of SEQ ID NO 2 in that it additionally comprises the sequence downstream of the RBS to include the ATG-codon of the *rpsF* gene.

As can be seen from figure 3, the DNA sequence located upstream of the –35 region comprises several AT-rich regions which will render the DNA helix more flexible. This recurrent motive is a typical DNA bending motive.
Such DNA-bending motives make it possible for distant upstream sequences involved in transcription, to become spatially located close to the promoter region.
As shown in figure 5, several constructs have been made, in which the *rpsF*-promoter according to the invention is fused to a promoter-less ß-galactosidase gene, and is preceded by stretches of nucleotides, of different length, as present upstream of the promoter sequence in the native situation. Figure 6 clearly shows that those constructs having longer stretches of native nucleotide sequences

upstream the –35 region still in place give a higher expression level compared to those constructs having a shorter upstream –35 sequence.
This clearly shows, that the presence of these AT-rich regions enhances the efficiency of the *rpsF*-promoter according to the invention.

Therefore, in a more preferred form of this embodiment, the promoter described in the invention is preceded by a stretch of nucleotides having at least 1, preferably 2, more preferably even three AT-rich regions, such as e.g. ATTT, ATTTA or TTATT upstream of the –35 region.

Preferably, the stretch of nucleotides having AT-rich regions upstream of the –35 region comprises the nucleotide sequence as depicted in SEQ ID NO 4, more preferably SEQ ID NO 5, even more preferably SEQ ID NO 6.

The sequence of the *ywpH* promoter is given in figure 4. Actually, the region of this promoter that is of importance is referred to as a *comK*-box. *ComK*-boxes play an important role in the competence-signal-transduction network. *ComK*-boxes have as a common characteristic that they share the consensus motive AAAANNNNNTTTT. The following must be stressed here: a consensus motive is a motive statistically the most prevalent motive found, when several of these motives are compared. This does not mean that minor modifications are not tolerated. Occasionally, A/T-, A/G- or A/C-transitions are found in the AAAA stretch of the motive and occasionally, T/A-, T/G- or T/C-transitions are found in the TTTT-stretch. The TATT tetranucleotide found instead of the TTTT sequence in the most upstream located *comK*-box of the *ywpH* gene is a good example of such a transition. Therefore, a "*comK*-box" is understood to have a sequence that does not differ at the "A"-side and/or the "T"-side of the box in more than one nucleotide compared to the consensus sequence. As a result, a *comK*-box as used in the present invention, i.e. falling under the definition of a consensus *comK*-box, could e.g. have the sequence AAAANNNNNTTTT, ATAANNNNNTTTT, AATANNNNNTATT or AAAANNNNNTATT. These examples are of course not limiting.

Another characteristic of *comK*-boxes is that they are always found twice. The presence of two *comK*-boxes is a prerequisite for their functionality.
The two *comK*-boxes in figure 4 are presented in bold letters.

The length of the spacer region separating the two *comK*-boxes is not critical. Values between 10 and 150 base pairs are however quite commonly found.

The two *comK*-boxes are located upstream of the gene to be expressed at a distance that is not critical. Roughly however, the distance between the *comK*-boxes and the gene to be expressed should not exceed 500 nucleotides. Typically, the distance would be between 20 and 200 nucleotides.

More information about the *comK*-boxes can be found e.g. at http://elmo.ims.u-tokyo.ac.jp/dbtbs/tfac/ComK.html.

Therefore, another embodiment of the invention relates to an expression cassette that comprises a promoter having two *comK*-boxes with a consensus sequence AAAANNNNNTTTT that are present twice within a mutual distance of between 10 and 150 nucleotides. Thus the sequence of this kind of promoter can be represented shortly as follows: AAAANNNNNTTTTX$_n$AAAANNNNNTTTT, wherein X$_n$ represents a number of nucleotides between 10 and 150, and N and X can be A, T, G or C.

In a preferred form of this embodiment, the expression cassette according to the invention comprises the promoter of the *ywpH* gene.
This promoter comprises the nucleotide sequence as depicted in SEQ ID NO: 7.

In a more preferred form of this embodiment, the promoter is followed by the native downstream nucleotide sequence up to, and including, the *ywpH* RBS. This promoter has the sequence as depicted in SEQ ID NO: 8.

In an even more preferred form of this embodiment, the promoter is followed by the native downstream nucleotide sequence up to, and including, the *ywpH* RBS and further extending to the start codon of the *ywpH* gene. This promoter has the sequence as depicted in SEQ ID NO: 9.

Another embodiment of the invention relates to recombinant plasmids that comprise an expression cassette according to the invention. As mentioned above, such plasmids serve as a way of transforming the host cell. Such plasmids may be high-copy or low-copy plasmids.
Techniques for the transformation of bacterial cells, more specifically *Bacillus*-like cells are well-known in the art.

If desired, 5'- terminal and 3'-terminal parts of a bacterial gene encoding an e.g. non-essential gene can be added at the upstream and downstream end of the expression cassette according to the invention. This allows for homologous recombination with the host cell's genome once the expression cassette optionally carried by the recombinant plasmid is brought into the host cell. The expression cassette will thus become stably integrated into the host's genome. Homologous recombination into a selectable non-essential gene highly facilitates the selection for integrants. As a selectable non-essential gene in *B. subtilis*, the *amyE* gene would be a very suitable gene.

Still another embodiment of the invention relates to bacterial host cells comprising an expression cassette or a recombinant plasmid according to the invention. As mentioned above, this embodiment relates to both bacterial host cells comprising an expression cassette located on a recombinant plasmid and bacterial host cells comprising an expression cassette integrated in the bacterial genome.

Again still another embodiment of the invention relates to promoters as described above for use in an expression cassette.

And still another embodiment of the invention relates to promoters as described above for use in a heterologous expression system.

Again another embodiment of the invention relates to methods for the heterologous expression of a gene wherein those methods comprise the construction of an expression cassette according to the present invention. Examples of the construction of such an expression cassette are given in the Examples below.

Still other embodiments relate to the use of a promoter as described in the present invention in an expression cassette and to the use of such a promoter in a heterologous expression system.

Finally, an embodiment of the present invention relates to methods for the heterologous expression of a gene, that comprise the transformation of a bacterium with an expression cassette according to the invention.

Example 1

Transcription analysis of the *ssb* and *ywpH* genes.

For all PCR-reactions *B. subtilis* 168 DNA was used as template. This strain can be obtained from the Bacillus Genetic Stock Center; Strains & Data, The Ohio State University, Department of Biochemistry, 484 West Twelfth Avenue, Columbus, Ohio 43210, USA.

Expression of these two genes and localization of their respective promoters. Transcriptional fusions of the potential promoter-containing fragments with a promoter less *lacZ* gene were constructed and integrated into the *B. subtilis* chromosome at an ectopic site: the alpha-Amylase E gene, *amyE*.
Upstream regions of the *ssb* and *ywpH* genes, fragment S1 (from 295 bp upstream to 11 bp downstream of the start codon of *ssb*), S2 (from 602 bp upstream to 11 bp downstream of the start codon of *ssb*), R1 (from 274 bp upstream to 50 bp downstream of the start codon of *rpsF*), Y1 (from 282 bp upstream to 10 bp downstream of the start codon of *ywpH*) were amplified by PCR using Pwo DNA polymerase (Roche) and chromosomal DNA of *B. subtilis 168* as template. The PCR fragments were cloned into the SmaI-digested promoter-screening vector pBTK2 (pBTK2 is a plasmid that carries 3'- and 5'-terminal parts of the *Bacillus subtilis amyE* gene, each flanking one side of a promoter less *lacZ* gene. See for details: Meijer, W. J. J. 1995. Replication and maintenance of plasmids in *Bacillus subtilis*. Thesis, available through the University of Groningen, The Netherlands). Resulting plasmids carrying the insert in the correct orientation were linearized and used to transform *B. subtilis 168,* selecting for kanamycin resistant transformants. The transformants were screened for an amylase-deficient phenotype to confirm that the construct had integrated at the *amyE* locus. The cloning and transformation were performed according to common techniques (Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., the supplier's manuals, and Bron, S. and G. Venema. 1972. Mutat.Res. 15:1-10).
The blue or white phenotypes of the resulting strains BIV7 (S1), 17 (S2), *8 (RI),* and 12 (Y1) on rich (TY) and minimal medium (MM) agar plates (Meima, R., C. Eschevins, S. Fillinger, A. Bolhuis, L. W. Hamoen, R. Dorenbos, W. J. Quax, J. M. van Dijl, R. Provvedi,1. Chen, D. Dubnau, and S. Bron. 2002. J.Biol.Chem. 277: 6994-7001), respectively, containing 0.004 % X-Gal revealed promoter activity only

for the constructs in strain BIV 17, *8,* 12 and 13, but not in BIV7. In BIV12, promoter activity was detected only in MM indicating a medium-dependent expression of the gene *ywpH* (Figure 1). Whereas *ywpH* was found to be transcribed from a promoter directly upstream of the gene, no promoter activity could be detected from the S 1 fragment comprising the 295 bp immediately upstream of the *ssb* start codon. However, promoter activity was detected from the S2 fragment containing also the complete *rpsF* gene and 274 bp upstream of its start codon indicating that *ssb* is the second gene of an operon, which is constituted of the *rpsF*, *ssb,* and *rpsR* genes. Clearly promoter activity was found also from the smaller RI fragment comprising the 274 bp upstream of the *rpsF* start codon only. These results suggest that *ssb is* cotranscribed and co-regulated with genes coding for ribosomal proteins, thereby coupling the regulation of protein synthesis and DNA metabolism.

In order to study the expression of *ssb* and *ywpH* in more detail, strains containing the S1-, R1- and Y1-lacZ fusions (BIV7, *8* and 12), respectively, were grown in TY and MM and ß-galactosidase activity was examined as described by Miller et al. (Miller, J. H. 1982. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Whereas no expression could be detected from the S1 fragment under the conditions employed, the *ssb* operon appeared to be strongly expressed from the *rpsF*-promoter in both rich and minimal media. The highest values (between 200 and 300 Miller units per OD) were reached during exponential growth (Figure 2A, B). When cells stopped growing the expression dropped. There is still expression in the stationary phase but on a much lower level.

In conclusion these results show directly growth-related characteristics of expression behavior of the *ssb* operon, and thus of the *rpsF*-promoter.

In contrast, no expression of the *ywpH* gene could be detected in cells of *B. subtilis* BIV12 grown in TY and only very low expression was observed in exponentially growing cells in MM. However, expression of *ywpH* increased significantly, when cells growing in MM entered the stationary phase and reached its highest level within two hours thereafter (Figure 2C).

In conclusion: the *ywpH* promoter is only expressed during the stationary phase in MM.

Below, the primers for, and the sequences of the S1, S2, R1 and Y1 constructs are given. Underlined regions reflect restriction sites.

S1: made with primers

ssb-1 Forward primer to amplify the *ssb* promoter region GCGAAGCTTCCAAACATTGACGAAGAGTCT

ssb-2 Reverse primer to amplify the *ssb* promoter region GCTGGATCCTCGGTTAAGCATAAGAAAGACC

Sequence:

GCTTCCAAACATTGACGAAGAGTCTAAAAAAGCAGTTATCGAGCGTTTCAACAAC
GTTCTGACTTCTAACGGTGCGGAGATCACTGGAACAAAGGATTGGGGTAAACGT
CGTCTTGCTTACGAAATCAACGATTTCCGCGACGGTTTCTACCAAATCGTAAACG
TTCAATCTGACGCTGCGGCAGTTCAAGAATTTGACCGTCTAGCTAAGATCAGTG
ACGATATCATTCGCCACATTGTTGTTAAAGAAGAAGAATAAGCAATTTTGAAATAT
ATAATGGTAAAAGGTGGTCTTTCTTATGCTTAACCGAGGAT

S2: made with primers

rps-1 Forward primer to amplify the *rpsF* promoter region GCGAAGCTTGTGACTTTGAGCGGGGCTT

Ssb-2 Reverse primer to amplify the *ssb* promoter region GCTGGATCCTCGGTTAAGCATAAGAAAGACC

Sequence:

GCTTGTGACTTTGAGCGGGGCTTCATTCGTGCTGAGACAGTTGCTTATGAGGAT
CTTCTTGCGGGCGGCGGTATGGCAGGAGCTAAAGAGGCAGGAAAAGTCCGCCT
TGAAGGGAAAGAATATGTGGTCCAAGACGGAGATGTTATTCATTTCCGATTTAAT
GTATAGGATGCAGTTGTAAAGGGACAAGAGCTTTGGTATAATATAAAATTGTGAG
TAATAGAATTATTGCTCCTTGCCCATTATGGGCCGCTTAGTCCAAAAGGAGGTG
CAAACAGATGAGAAAGTACGAAGTTATGTACATTATCCGCCCAAACATTGACGAA
GAGTCTAAAAAAGCAGTTATCGAGCGTTTCAACAACGTTCTGACTTCTAACGGTG
CGGAGATCACTGGAACAAAGGATTGGGGTAAACGTCGTCTTGCTTACGAAATCA
ACGATTTCCGCGACGGTTTCTACCAAATCGTAAACGTTCAATCTGACGCTGCGG
CAGTTCAAGAATTTGACCGTCTAGCTAAGATCAGTGACGATATCATTCGCCACAT
TGTTGTTAAAGAAGAAGAATAAGCAATTTTGAAATATATAATGGTAAAAGGTGGT
CTTTCTTATGCTTAACCGAGGAT

R1: made with primers:

Rps-1 Forward primer to amplify the *rpsF* promoter region GCGAAGCTTGTGACTTTGAGCGGGGCTT

Rps-2 Reverse primer to amplify the *rpsF* promoter region GCTGGATCCATCTTCGTCAATGTTTGGGCG

Sequence:

GCTTGTGACTTTGAGCGGGGCTTCATTCGTGCTGAGACAGTTGCTTATGAGGAT
CTTCTTGCGGGCGGCGGTATGGCAGGAGCTAAAGAGGCAGGAAAAGTCCGCCT
TGAAGGGAAAGAATATGTGGTCCAAGACGGAGATGTTATTCATTTCCGATTTAAT
GTATAGGATGCAGTTGTAAAGGGACAAGAGCTTTGGTATAATATAAAATTGTGAG
TAATAGAATTATTGCTCCTTGCCCATTATGGGCCGCTTAGTCCAAAAGGAGGTG
CAAACAGATGAGAAAGTACGAAGTTATGTACATTATCCGCCCAAACATTGACGAA
GATGG

Y1: made with primers

ywpH-1 Forward primer to amplify the *ywpH* promoter region CCCAAGCTTTCAAGCTGTCAATGCCG

ywpH-2 Reverse primer to amplify the *ywpH* promoter region CGCGGATCCGATTGAACATGCGATTCC

Sequence:

GCTTTCAAGCTGTCAATGCCGGAAAAAAAATTGAGCTTTCAGTGGTTTGCGTGG
GATGGCTCTTCCTATGTGCGCATGAATACGCAAAACTGGCTGACAAAGCAAATC
TTTTTCCGTTTTTTAAAAAGTACATATTTCTTCAAAGGAAAAAAGCAAAAGATGTT
TTTAGCTGAAGGAAAAATGAAAACGAAAGATAAAAACAGAGGCTGAAAGCCATTT
TTAAGCGTTTTTCTTTTCTTGTTGCATCATTTACAATACATACAACCGCAAGGAGA
GGAGGAATCGCATGTTCAATCGGAT

Example 2

Cloning strategy *Bacillus subtilis* integration/ multi copy plasmid with expression cassette.

In this construct, the *rpsF* promoter as described in the invention is used, but the RBS is the *spoVG* RBS as also found on the *Bacillus subtilis* genome. The gene cloned and expressed in this expression cassette is the *Clostridium perfringens* beta-toxin (Hunter et al.; Infect. & Immun. 61: 398-965 (1993))

1 PCR reactions:

1.1 amyE

Primers for amplifying the *amyE* gene introducing a 5' *XbaI* site and a 3' *SacI* site were as follows:

amyE-1: Forward primer: GCTCTAGACGAATTATATGGATGTGAC

amyE-2: Reverse primer: GCCTAGAGCTCGGATCTCCTTTTCCGATTG

Template: chromosomal DNA 168

Product size: 823 bp

1.2 Kanamycin resistance gene

Primers for amplifying the kanamycin resistance gene (same as in pUB110) introducing 5' *PstI* and *NruI* sites and a 3' kpnI site

Kn-1: Forward primer: CAATCTGCAGTCGCGATGAGAATAGTGAATGGAC

Kn-2: Reverse primer: CGGGTACCTCAAAATGGTATGCGTTTTG

Template: pUB110 (Gryczan, T. J., Contente, S., Dubnau, D.; J. Bacteriol. 134: 318-329 (1978), McKenzie, T., Hoshino, T., Tanaka, T., Sueoka, N.; Plasmid 15: 93-103 (1986)).

Product size: 794 bp

1.3 *spoVG* RBS

Artificially made:

GAAGATCTCTCGAGGGTACCTTGATACACTAATGCTTTTATATAGGGAAAAGGTGGTGAACTCATATGAATCGAG
CTTCTAGAGAGCTCCCATGGAACTATGTGATTACGAAAATATATCCCTTTTCCACCACTTGAGTATACTTAGCTC

GAAGATCTCTCGAGGGTACCTTGATACAC CTAATGCTTTTATATAGGGAAAAGGTGGTG
CATGGAACTATGTGATTACGAAAATATATC CCTTTTCCACCACTTGAGTATACTTAGCTC

Primers:

*spoVG*1 5'- GAAGATCTCTCGAGGGTACCTTGATACAC-3'

*spoVG*2 5'- CTATATAAAAGCATTAGTGTATCAAGGTAC-3'

*spoVG*3 5'-CTAATGCTTTTATATAGGGAAAAGGTGGTG-3'

*spoVG*4 5'-CTCGATTCATATGAGTTCACCACCTTTTCC-3'

spoVG RBS can be found on the *Bacillus subtilis* genome.

Because of a *BglII* site in this kanamycin gene, the *spoVG* RBS could not be cloned with *BglII*, so instead of *BglII* a *PstI* site must be introduced:

*spoVG*5: Forward primer: GACTGCAGCTCGAGGGTACCTTGATACAC

*spoVG*4: Reverse primer: CTCGATTCATATGAGTTCACCACCTTTTCC

Template: s*poVG* RBS

Product size: 75 bp

1.4 gnt terminator

Primers for amplifying 3 stop codons and the gnt-terminator introducing 5' *BamHI* and *HpaI* sites and 3' *XbaI* and *StuI* sites.

Gntterm-1: Forward primer:

GCGGATCCAGGCCTAACTAATTAACCTGTATTAAAAACACG

Gntterm-2: Reverse primer: GCTCTAGAGTTAACCTTCTGTTGTTTGGGATAG

Template: chromosomal DNA 168

Product size: 107 bp.

1.5 *Clostridium perfringens* beta-toxin (Btox)

Primers for amplifying Btox, introducing a 5' *PstI*, *NdeI* site and a 3' *BamHI* site.

B-toxoid3: 5'-AACTGCAGAGATCTCATATGAAGAAAAAATTTATTTCATT-3'

Btoxoid2: CGCGGATCCTTAAATAGCTGTTACTTTGTGAG

Product size: About 1000 bp

1.6 The *rpsF* promoter without *rpsF* RBS

rpsF-3: 5'-GGAATTCCTGCAGGTGACTTTGAGCGGGGCTTC-3'

rpsF-4: 5'-CGTACTTTCTCATATGTTTGCACC-3'

Template: chromosomal DNA 168

Product size: 295 bp.

2 Cloning:

2.1 Cloning kanamycin in pBlueSK-

The kanamycin PCR product was digested with *PstI* and *KpnI* and ligated into the likewise digested pBlueSK-. This resulted in pIV26.

2.2 Cloning *amyE* in pIV26

The *amyE* PCR product was subsequently digested with *XbaI* and *SacI* and ligated into the likewise digested pIV26. This resulted in pIV27.

2.3 Cloning Btox in pIV27

The *Btox* PCR product was digested with *PstI* and *BamHI* and ligated into the likewise digested pIV27. This resulted in pIV28.

2.4 Cloning the gnt terminator in pIV28

The gnt terminator PCR fragment was digested with *XbaI* and *BamHI* and ligated into likewise digested pIV28, which resulted in pIV29.

2.5 Cloning the *spoVG* RBS in pIV29

The *spoVG* PCR product was digested with *PstI* and *NdeI* and ligated into the likewise digested pIV29. This resulted in pIV33.

2.6 Cloning the *spoVG*-RBS + Btox + terminator- cassettes in pTRKH2

Plasmid pIV33 was digested with *XbaI* and *PstI* and ligated into likewise digested pTRKH2. This resulted in pIV69. pTRKH2 is described by O'Sullivan, D.J. and Klaenhammer, T,R. in Gene 137: 227-231 (1993).

2.7 Cloning the *rpsF* promoter (with *spoVG* RBS) in pIV69

Plasmid pIV69 was digested with *StuI* and ligated to the blunt ended *rpsF* PCR fragment. This resulted in pIV82.
This plasmid was used for transformation of *Bacillus subtilis*.

Bacillus subtilis was subsequently transformed with the construct of 2.7, and grown in rich medium. As is clear from figure 7, large amounts of *Clostridium perfringens* beta-toxin were expressed and excreted into the supernatant. The increase in formation of insoluble material in the cell is probably due to too high a level of expression. The expression level can easily be decreased by using a low copy vector, or by

integration of the expression cassette in the genome at a single integration site or at a few integration sites.

So far, several other toxins, i.a. the Pasteurella multocida toxin (see i.a. EP409895), the E. coli heat-labile toxin and the Staphylococcus aureus alfa-toxin have equally successfully been expressed.

This shows that the expression cassettes according to the invention are very versatile expression vectors.

Example 3:

Cloning strategy double integration construct

1. **PCR**

1.1 *amyE*back:

PCR for amplifying the 5' *amyE* region, introducing a HindIII and a XhoI site.

*amyE*back-1: CCCAAGCTTTCGACATGGATGAGCGATG

*amyE*back-2: GCAGCTCGAGGCTCCGGCGCAAATGCAG

Product size: 1256 bp

1.2 *amyE*front:

PCR for amplifying the 3' *amyE* region, introducing a SacI and a XbaI site.

*amyE*front-1: CGCGAGCTCAACAAAATTCTCCAGTCTTC

*amyE*front-2: CGGTCTAGAAGTTTTTAATTTGTGTGTTTCC

Product size: 634 bp

1.3 Chloramphenicol:

PCR for amplifying the chloramphenicol resistance gene, introducing a EcoRI and a HindIII site.

cmR-1: CGGGAATTCTCATGTTTGACAGC

cmR-2: CGCGAAGCTTCCCAGTAGTAGGTTGAGGCC

Product size: 1310 bp

1.4 Terminator:

PCR for amplifying stop codons in all frames and the gnt terminator sequence, introducing BamHI, SnaBI, HpaI and XbaI sites.

Gntterm-3: Forward primer:

GCGGATCCTACGTAAACTAATTAACCTGTATTAAAAACACG

Gntterm-2: Reverse primer: GCTCTAGAGTTAACCTTCTGTTGTTTGGGATAG

Template: chromosomal DNA 168

Product size: 107 bp.

1.5 *rpsF* promoter:

PCR for amplifying the *rpsF* promoter, introducing a PstI and a NdeI site.

rpsF-3: 5'-GGAATTCCTGCAGGTGACTTTGAGCGGGGCTTC-3'

rpsF-5 5'-CGTACTTTCTCATATGTTTGCACC-3'
Template: 168 chromosomal DNA
Product size: 295 bp

1.6 Btox:
PCR for amplifying Btox, introducing a BamHI and NdeI site.
B-toxoid3: AACTGCAGAGATCTCATATGAAGAAAAAATTTATTTCATT
Btoxoid2: CGCGGATCCTTAAATAGCTGTTACTTTGTGAG
Product size: 1037 bp

2 Cloning

2.1 *amyE*back in pBlueKS-
The *amyE*back PCR product was digested with HindIII and XhoI and ligated into the likewise digested pBlueKS- to produce construct 1.

2.2 *amyE*front in construct 1.
The *amyE*front PCR product was digested with SacI and XbaI and ligated into likewise digested 1 to produce construct 2.

2.3 Chloramphenicol in construct 2.
The cm PCR product was digested with EcoRI and HindIII and ligated in likewise digested 2 to produce construct 3.

2.4 gnt terminator in construct 3.
The terminator PCR product was digested with BamHI and XbaI and ligated in likewise digested 3 to produce construct 4.

2.5 *rpsF* promoter in construct 4.
The *rpsF* PCR product was digested with PstI and ligated into PstI-SmaI digested 4 to produce construct 5.

2.6 Btox in construct 5.
The Btox PCR product was digested with BamHI and NdeI and ligated into likewise digested 5 to produce construct 6.

Legend to the figures.

Figure 1: Gene organization of the *ssb* operon (A) and the *ywpH* gene (B) in *B. subtilis* 168 with schematic representation of the constructed *lacZ* fusions. Black lines in bold type represent the PCR-amplified DNA fragments fused to the promoter less *lacZ* gene.
The presence or absence of β-galactosidase activity in the various constructs after growth in rich (TY) or minimal medium (MM) is indicated on the right site by a '+' and '-', respectively.

Figure 2: Expression of the *ssb* operon and the *ywpH* gene in *B. subtilis*.
Bacterial growth (open symbols) in TY (triangles) or minimal medium (circles) and expression of the transcriptional *lacZ* fusions (closed symbols) under the control of the *rpsF* promoter, in the strain BIV8 (A, B) or the *ywpH* promoter in the strain BIV12 (C) reflected as β-galactosidase activity per OD.

Figure 3: upstream region of the *rpsF* gene, indicating the location of the –10 and –35 regions and AT-rich stretches further upstream, as well as the RBS and the start codon of the *rpsF* gene.

Figure 4: upstream region of the *ywpH* gene, indicating the location of the two *comK* boxes (bold), the RBS (italic) and the 30 first nucleotides (including ATG start codon) of the *ywpH* gene (underlined).

Figure 5: schematic representation of constructs of various length comprising the *rpsF* promoter fused to the ß-galactosidase gene. The location of the –10 and –35 region, the RBS and the *rpsF* start codon as well as the length of the various upstream fragments are indicated.

Figure 6: schematic representation of the ß-galactosidase activity of the various constructs of figure 5.

Figure 7: Expression of Btox under the control of *rpsF* promoter on a high copy vector. Sup=supernatant, Sol=soluble fraction, Insol=insoluble fraction. BtoxCp=wild type Clostridium perfringens beta-toxin. T=growth time in hours.

Claims

1) Expression cassette, characterized in that said expression cassette comprises a promoter obtainable from the region between the stop codon of the *yyaF* gene and the start codon of the *rpsF* gene as found on the *Bacillus subtilis* genome.

2) Expression cassette, characterized in that said expression cassette comprises a promoter having the following characteristics
a) a nucleotide sequence TATAAT, preferably GTATAAT or TATAATA at the –10 site,
b) a nucleotide sequence TTGTAA, preferably GTTGTAA or TTGTAAA at the –35 site,
c) a spacer of 17 +/- 2 nucleotides, preferably 17 +/- 1 nucleotides, more preferably 17 nucleotides.

3) Expression cassette according to claim 1 or 2, characterized in that the promoter comprises the nucleotide sequence as depicted in SEQ ID NO 1.

4) Expression cassette according to claim 1 or 2,, characterized in that the promoter comprises the nucleotide sequence as depicted in SEQ ID NO 2.

5) Expression cassette according to claim 1 or 2, characterized in that the promoter comprises the nucleotide sequence as depicted in SEQ ID NO 3.

6) Expression cassette according to claims 1-5, characterized in that the promoter is preceded by a stretch of nucleotides having at least 1, preferably 2, more preferably even three AT-rich regions upstream of the –35 region.

7) Expression cassette according to claim 6, characterized in that said stretch of nucleotides having AT-rich regions upstream of the –35 region comprises the nucleotide sequence as depicted in SEQ ID NO 4, preferably in SEQ ID NO 5, more preferably in SEQ ID NO 6.

8) Expression cassette, characterized in that said expression cassette comprises a promoter having a consensus sequence

AAAANNNNNTTTTX$_n$AAAANNNNNTTTT, wherein 10<n<150

9) Expression cassette according to claim 8, characterized in that the promoter comprises the nucleotide sequence as depicted in SEQ ID NO: 7, preferably in SEQ ID NO: 8, more preferably in SEQ ID NO: 9.

10) Recombinant plasmid characterized in that said plasmid comprises an expression cassette according to claims 1-9.

11) Bacterial host cell comprising an expression cassette according to claims 1-9 or a recombinant plasmid according to claim 10.

12) Use of a promoter as described in claims 1-9 in an expression cassette.

13) Use of a promoter as described in claims 1-9 in a heterologous expression system.

14) Method for the heterologous expression of a gene, characterized in that said method comprises the construction of an expression cassette according to claims 1-9.

15) Method for the heterologous expression of a gene, characterized in that said method comprises the transfection of a bacterium with an expression cassette according to claims 1-9.

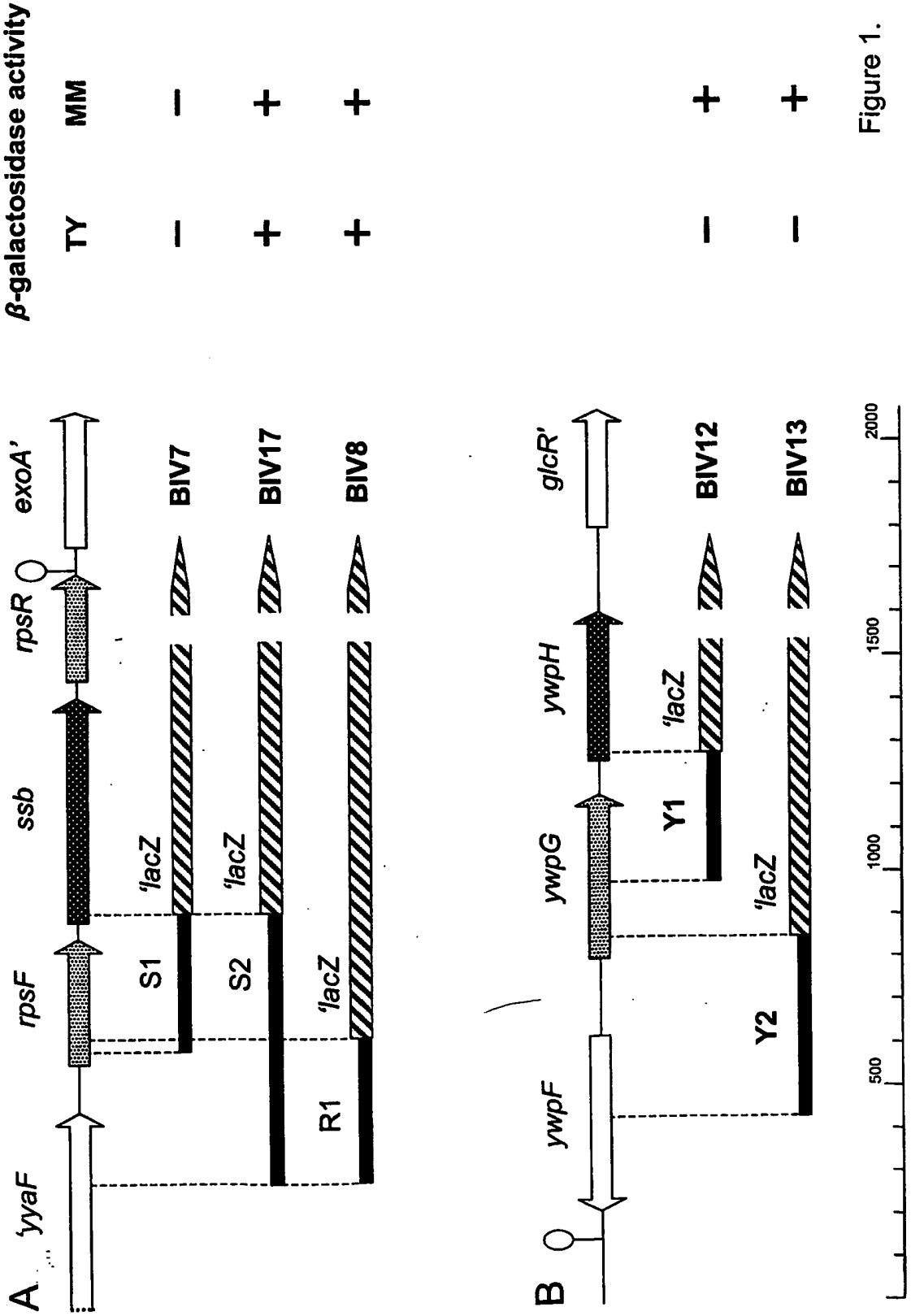

β-galactosidase activity
TY
MM
BIV7
BIV17
BIV8
BIV12
BIV13
A
B
'yyaF
rpsF
ssb
rpsR
exoA'
'lacZ
S1
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glcR'
Y1
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500
1000
1500
2000

Figure 1.

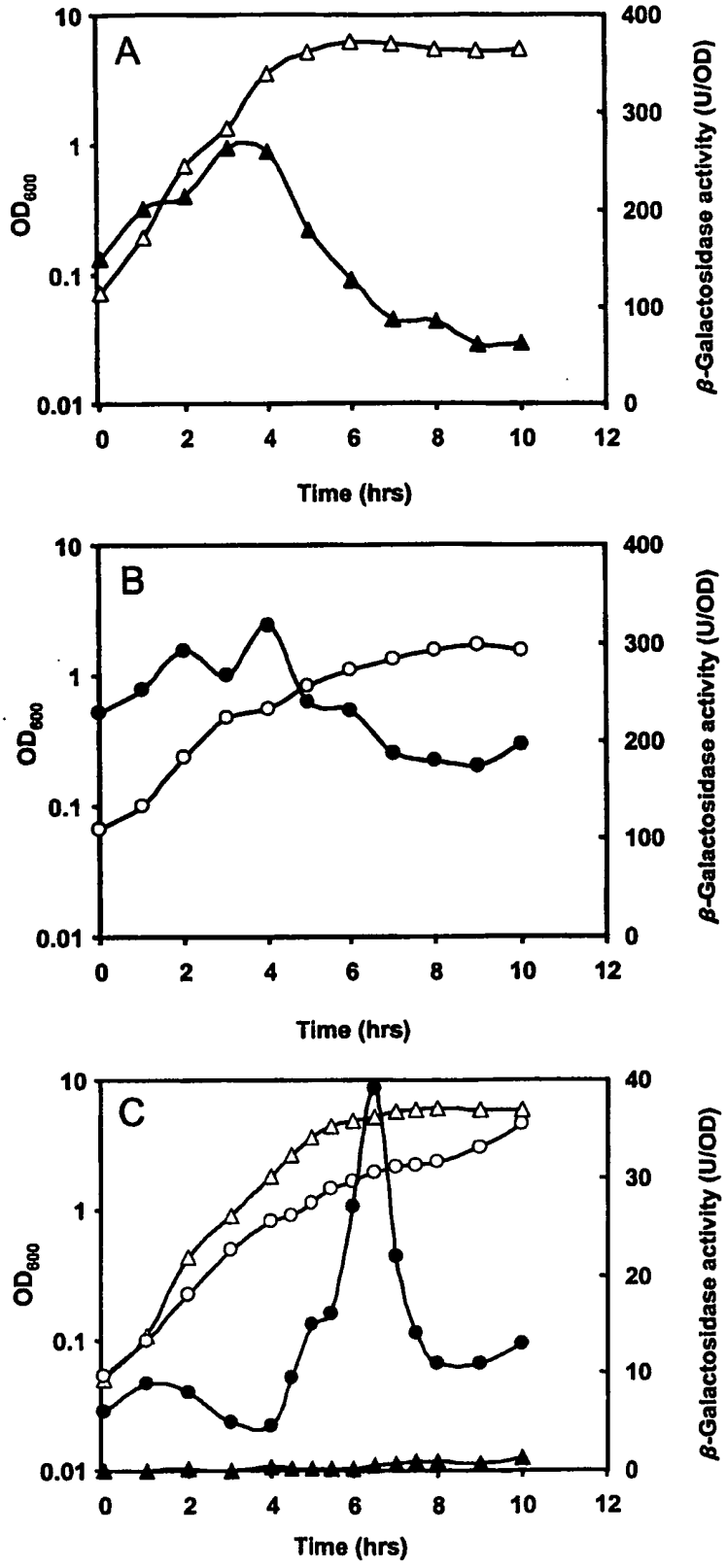

A
B
C
OD_{600}
β-Galactosidase activity (U/OD)
Time (hrs)
10
1
0.1
0.01
400
300
200
100
0
40
30
20
10
0
2
4
6
8
10
12

Figure 2.

CTGAGTGTGCAGGAATTATTCATAGTGACTTTGAGCGGGGCTTCA
Begin promoter construct

TTCGTGCTGAGACAGTTGCTTATGAGGATCTTCTTGCGGGCGGCG

GTATGGCAGGAGCTAAAGAGGCAGGAAAAGTCCGCCTTGAAGGGA

AAGAATATGTGGTCCAAGACGGAGATGTTATTCATTTCCGATTTA
Stopcodon yyaF - 35 - 10
ATGTATAGGATGCAGTTGTAAAGGGACAAGAGCTTTGGTATAATA

TAAAATTGTGAGTAATAGAATTATTGCTCCTTGCCCATTATGGGC
RBS Start codon *rpsF*
CGCTTAGTCCAAAAGGAGGTGCAAACAGATGGAAGTTACTGACGT

Figure 3.

TCAAGCTGTCAATGCCGGAAAAAAAATTGAGCTTTCAGTGGTT
TGCGTGGGATGGCTCTTCCTATGTGCGCATGAATACGGCAAAA
CTGGCTGACAAAGCAAATCTTTTTTCCGTTTTTTA**AAAA**GTACA
TATTCTTCAAAGGAAAAAAAGC**AAAA**GATGT**TTTT**AGCTGAAG
GAAAAATGAAAAACGAAAGATAAAAACAGAGGCTGAAAGCCATT
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Figure 4.

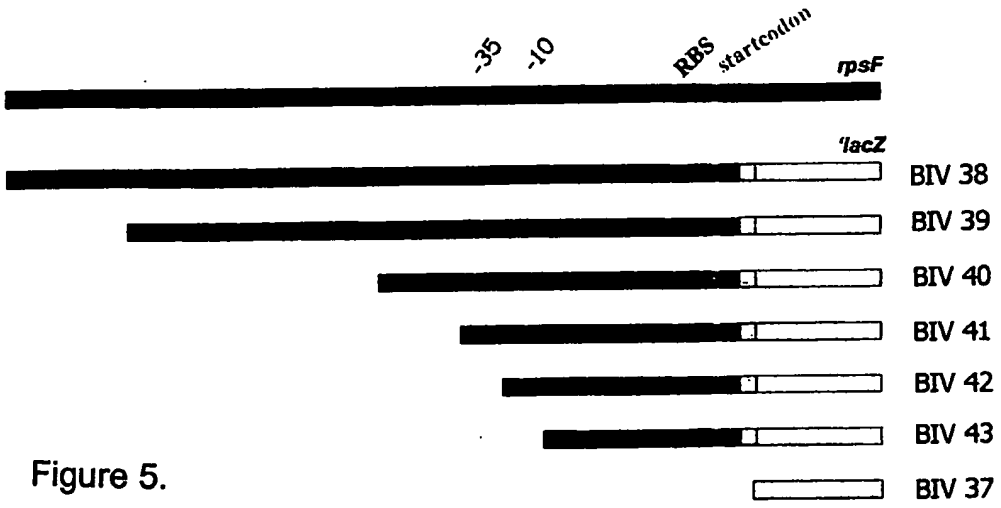


Figure 5.

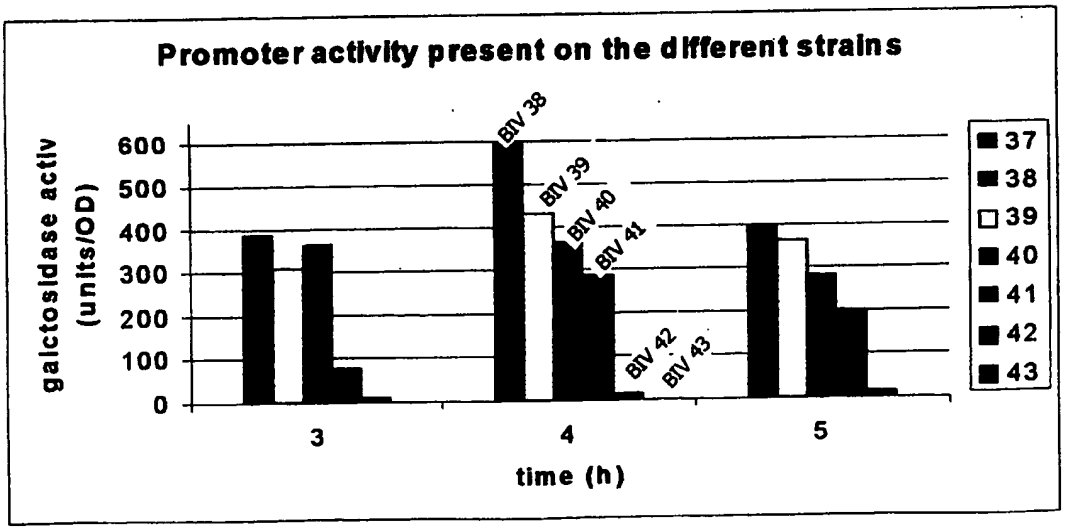


Figure 6.

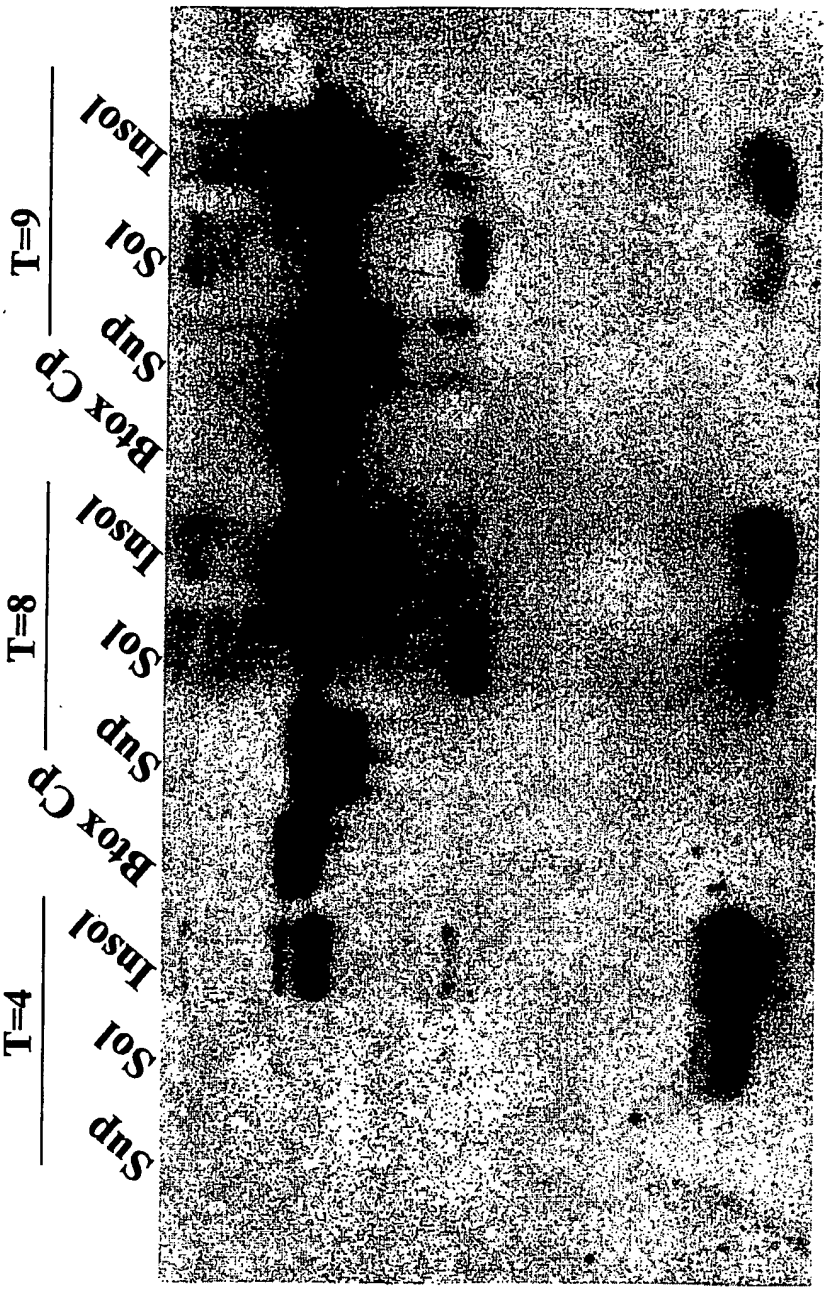

T=4
Snp
Sol
Insol
Btox Cp
T=8
Snp
Sol
Insol
Btox Cp
T=9
Snp
Sol
Insol

Figure 7.

DT05 Rec'd PCT/PTO 04 FEB 2005

SEQUENCE LISTING

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<120> novel bacterial expression system

<130> 2002.012

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/08506

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/75

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL [Online] EBI; 12 July 1994 (1994-07-12), "B. subtilis sigma factor 43 recognised strong promoter sequence." XP002245464 Database accession no. X54036 abstract -----	1,3-7, 10-12
X	DATABASE EMBL EBI; 17 April 1994 (1994-04-17), "B. subtilis 168 clone BJ29 promoter region." XP002245465 abstract -----	1,3-7, 10-12
A	US 5 726 042 A (PAUL LELAND SHAWN ET AL) 10 March 1998 (1998-03-10) ----- -/--	1,3-7, 10-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 2003	13. 01. 2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mata Vicente, T.

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LAM K H E ET AL: "Construction of an efficient Bacillus subtilis system for extracellular production of heterologous proteins" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 63, no. 3, 27 August 1998 (1998-08-27), pages 167-177, XP004154133 ISSN: 0168-1656 abstract ----	1,3-7, 10-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/08506

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

(1, 3-7) - completely; (10-15) - partially

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: (1, 3-7) - completely; (10-15) - partially

Expression cassette, characterized in that it comprises a promoter obtainable from the region between the stop codon of the yyaF gene and the start codon of the rpsF gene as found on the B. subtilis genome, wherein said promoter comprises SEQ ID NOs:1, 2 or 3. Plasmid comprising said expression cassette, host cell comprising it and use thereof in a heterologous expression system.

2. claims: 2 - completely; (10-15) - partially

Expression cassette, characterized in that it comprises a promoter having the following characteristics: a) a nucleotide sequence TATAAT at the -10 site; b) a nucleotide sequence TTGTAA at the -35 site; and c) a spacer of 17 +/- 1 nucleotides, more preferably 17 nucleotides. Plasmid comprising said expression cassette, host cell comprising it and use thereof in a heterologous expression system.

3. claims: (8,9) - completely; (10-15) - partially

Expression cassette, characterized in that it comprises a promoter having a consensus sequence AAAANNNNNTTTTXnAAAANNNNNTTTT, wherein said promoter comprises SEQ ID NOs:7, 8 or 9. Plasmid comprising said expression cassette, host cell comprising it and use thereof in a heterologous expression system.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 03/08506

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5726042 A	10-03-1998	NONE	